

Dual sgRNA-based Targeted Deletion of Large Genomic Regions and Isolation of Heritable Cas9-free Mutants in *Arabidopsis*

Yu Jin and Sebastian Marquardt*

Copenhagen Plant Science Centre, Department of Plant and Environmental Sciences, University of Copenhagen, Bülowsvej 21, 1870 Frederiksberg C, Denmark

*For correspondence: sebastian.marquardt@plen.ku.dk

[Abstract] CR/SPR/Cas9 system directed by a gene-specific single guide RNA (sgRNA) is an effective tool for genome editing such as deletions of few bases in coding genes. However, targeted deletion of larger regions generate loss-of-function alleles that offer a straightforward starting point for functional dissections of genomic loci. We present an easy-to-use strategy including a fast cloning dual-sgRNA vector linked to efficient isolation of heritable Cas9-free genomic deletions to rapidly and cost-effectively generate a targeted heritable genome deletion. This step-by-step protocol includes gRNA design, cloning strategy and mutation detection for *Arabidopsis* and may be adapted for other plant species.

Keywords: CR/SPR/Cas9, Cas9-free, dual-sgRNA, gBlock, Genomic deletion

[Background] Dual sgRNA-directed gene knockout by CR/SPR/Cas9 has been successfully used for genome editing in a variety of organisms (Wang *et al.*, 2013; Chen *et al.*, 2014; Char *et al.*, 2017; Cai *et al.*, 2018; Durr *et al.*, 2018; Cui *et al.*, 2019; Do *et al.*, 2019; Liu *et al.*, 2020). Targeted deletions of genomic DNA regions offer a valuable starting point for functional genomics studies (Hilton and Gersbach, 2015; Ford *et al.*, 2019; Gowthaman *et al.*, 2020). CR/SPR/Cas9-based methods to delete genomic regions benefit from two gRNAs flanking the target DNA regions (Xiao *et al.*, 2013; Canver *et al.*, 2014; Kistler *et al.*, 2015; Song *et al.*, 2016). In plants, one key bottleneck to perform multiplex gene targeting from a single transformation event is to include multiple gBlocks into one binary vector. A gBlock is composed of a RNA Polymerase III (RNAPIII) promoter, a gene specific sgRNA protospacer, a sgRNA scaffold and a RNAPIII terminator. However, gBlock DNA sequences are usually long and repetitive, rendering design expensive for synthetic DNA and laborious by traditional assembly methods (Gao *et al.*, 2016; Peterson *et al.*, 2016; Zhang *et al.*, 2016; Char *et al.*, 2017; Durr *et al.*, 2018; Pauwels *et al.*, 2018; Schuster, 2018; Wu *et al.*, 2018; Hui *et al.*, 2019; Fonseca *et al.*, 2020). For example, Durr *et al.* (2018) developed a dual-sgRNA vector by first modifying a pEN-Chimera entry vector to generate two gBlocks, then inserting two gRNAs into the modified entry vector by restriction enzymes, respectively, and finally cloning two gBlocks into the binary vectors. The multiple steps necessary are laborious and time-consuming. Although multiplex CR/SPR/Cas9 platforms by introducing repetitive gBlocks for targeted genome editing were reported (Ordon *et al.*, 2016; Schuster, 2018), several studies have noted that transformation of highly repetitive DNA sequences can trigger recombination and silencing of the RNA expression cassettes in a variety of species (Ma and Mitra, 2002; Lovett *et al.*, 2004; Brake *et al.*, 2008). To simplify targeted genomic regions and minimize potential recombination in *Arabidopsis*, we

combined and modified existing cloning-based assembly steps. First, we amplified the middle border of our target specific two-gBlocks from a previously developed vector pHEE2E-TRI (Wang *et al.*, 2015) in a single step. Second, we cloned the middle border into a known CRISPR/Cas9 binary vector pKIR1.1 (Maruyama *et al.*, 2013; Tsutsui and Higashiyama, 2017), allowing two-gBlocks with different Poll III-dependent promoters to reduce the repetitiveness. This vector harbors an RPS5A-Cas9 cassette driving high constitutive expression of Cas9 protein at all developmental stages including egg cells, thus achieves highly efficient mutation in the T1 generation of *Arabidopsis*. In addition, the expression cassette OLE1-tagRFP in this system shows red fluorescence in seeds, allowing rapid screening for heritable Cas9-free *Arabidopsis* mutants in the seed of primary transformants. We combined the advantages of the two vectors by a single PCR and a single cloning step, thus providing a simple and reliable protocol to generate stable inherited deletion mutants. Our strategy promises to save cost and time to delete any chromosomal region in *Arabidopsis*, and can be likely adapted for genome editing of multiple genes simultaneously. It also has the potential to simplify genomic deletion in other plant species.

Materials and Reagents

A. Consumables

1. Sterile pipette tips (Axygen, catalog numbers: TF-300-R-S [10 µl], T-350-C-L-R-S [300 µl], TF-1000-R-S [1,000 µl])
2. PCR microtubes (BioExpress, catalog number: T-3135-2)
3. 60 mm x 15 mm round Petri dishes (VWR, catalog number: 100488-404)

B. Competent cells

1. *Escherichia coli* HST08 competent cell (homemade, [protocol 1](#)), store at -80 °C
2. *Agrobacterium tumefaciens* GV3101 competent cells (homemade, [protocol 2](#)), store at -80 °C

C. Vectors

1. pHEE2E-TRI (Addgene, catalog number: 71288), store at -20 °C
2. pKIR1.1 (Addgene, catalog number: 85758), store at -20 °C

Note: Sequences of both vectors can be found in Addgene online.

pHEE2E-TRI sequence: <https://www.addgene.org/71288/sequences/>

pKIR1.1 sequence: <https://www.addgene.org/85758/sequences/>

D. Oligonucleotides 10 µM

Dual-sgRNA1_F: 5'-CACCTGCATACATTGN₂₀(protospacer 1)GTTTTAGAGCTAGAAATAGC-3'

Dual-sgRNA2_R: 5'-CACCTGCATACAAACN₂₀(protospacer 2 reverse complement)CAATCTCTTAGTCGA
CTCTAC-3'

Mlo 1938: 5'-TCCCAGGATTAGAATGATTAGG-3'

Primer_F: 5'-TTCTCTCTTCGCTCTCGTAG-3'
Primer_R: 5'-GGCCCAAATACTCTTCCAAGAC-3'
Cas9_F: 5'-CAGCCGACAAGAAAGTACAGC-3'
Cas9_R: 5'-ATGGTGGGGTACTTCTCGTG-3'

E. Enzymes and buffers

1. AarI (Thermo Fisher Scientific, catalog number: ER1581), store at -20 °C
2. T4 DNA Ligase (NEB, catalog number: M0202L), store at -20 °C
3. T4 Polynucleotide Kinase (NEB, catalog number: M0201L), store at -20 °C
4. T4 DNA Ligase Reaction Buffer (10x) (NEB, catalog number: B0202S), store at -20 °C
5. Alkaline Phosphatase, Calf Intestinal (CIP) (NEB, catalog number: M0290), store at -20 °C
6. Phusion High-Fidelity DNA Polymerase Kits (New England Biolabs, catalog number: M0530S), store at -20 °C
7. HotMaster Taq DNA Polymerase (VWR, catalog number: QUNT2200330), store at -20 °C
8. Wizard® SV Gel and PCR Clean-Up System (Promega, catalog number: A9282), store at room temperature
9. DNA plasmid kit (VWR, catalog number: D6943-02), store at room temperature

F. Reagents

1. MES (Sigma-Aldrich, catalog number: 4432-31-9), store at room temperature
2. KOH (Sigma-Aldrich, catalog number: 1310-58-3), store at room temperature
3. Sucrose (Sigma-Aldrich, catalog number: 57-50-1), store at room temperature
4. Plant agar (Sigma-Aldrich, catalog number: 9002-18-0), store at room temperature
5. Bacto agar (BD Biosciences, catalog number: 214030), store at room temperature
6. Bacto tryptone (BD Biosciences, catalog number: 211699), store at room temperature
7. Bacto yeast extract (BD Biosciences, catalog number: 212730), store at room temperature
8. Sodium chloride (Fisher Scientific, catalog number: 7647-14-5), store at room temperature
9. Murashige & Skoog medium (Duchefa Biochemie, catalog number: M524), store at 4 °C

G. Antibiotics

1. Spectinomycin (VWR, catalog number: 101454-196), store at -20 °C
2. Rifampicin (VWR, catalog number: 13292-46-1), store in a dry and well-ventilated place
3. Gentamycin (VWR, catalog number: 97062-974), store at 4 °C
4. Kanamycin (VWR, catalog number: 25389-94-09), store at 4 °C

H. Media (see Recipes)

1. LB liquid medium + 100 mg/L spectinomycin (store at 4 °C for one month)
2. LB agar plates + 100 mg/L spectinomycin (store at 4 °C for one month)
3. LB liquid medium + 100 mg/L spectinomycin + 20 mg/L rifampicin + 25 mg/L gentamycin + 25

mg/L kanamycin (store at 4 °C for one month)

4. LB agar plates + 100 mg/L spectinomycin + 20 mg/L rifampicin + 25 mg/L gentamycin + 25 mg/L
5. ½ MS-medium plates

Note: All media need to be autoclaved before adding antibiotics. For plates, 20 ml of media should be used per Petri dish.

Equipment

1. Pipettes (Thermo Scientific, Finnpipette™ F2, catalog numbers: 4642010 [0.2-2 µl], 4642030 [1-10 µl], 4642060 [2-20 µl], 4642080 [20-200 µl], 4642090 [100-1,000 µl])
2. Incubator (Thermo Scientific, catalog number: 51028132)
3. Shakers (Eppendorf, model: New Brunswick™ Innova® 44, catalog number: M1282-0000)
4. Stereo Fluorescence Microscope (Leica, model: M205FA)
5. Gel Doc EQ System (Bio-Rad, Universal Hood II, model: BGDII)
6. Heating blocks (Eppendorf, catalog number: T1317-1EA)
7. ThermoMixer® C (Eppendorf, catalog number: 5382000015)
8. PCR Thermo Cycler (Bio-Rad, model: T100, catalog number: 1861096)
9. Tabletop centrifuge (Thermo Fisher Scientific, catalog number: 75008801)
10. NanoDrop (Thermo Scientific, model: NanoDrop™ 2000C, catalog number: ND2000)
11. Agarose gel electrophoresis equipment (Bio-Rad, catalog number: 1704489EDU)
12. Plant growth chamber (photoperiod: 16 h light/ 8 h dark, temperature: 22 °C in the day/20 °C in the darkness, humidity: 65%, light intensity: 100 µE m⁻² s⁻¹)

Note: No equipment from specific manufacturers is required. Any equivalent device can be used.

Software

1. SnapGene® (SnapGene, <https://www.snapgene.com/>)
2. Image Lab Software (Bio-Rad, <https://www.bio-rad.com/>)

Procedure

A. Overview

The process that generates dual sgRNA-directed large deletion by CRISPR/Cas9 is shown in Figure 1. The vector pKIR1.1 can be ordered from Addgene. A gBlock is composed of a U6 promoter, a gene specific sgRNA protospacer, and a sgRNA scaffold and a terminator.

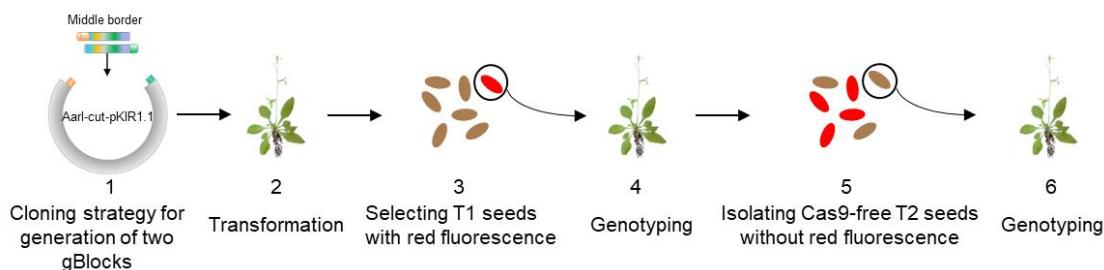


Figure 1. Flowchart for isolation of Cas9-free target mutants. 1. Cloning strategy for generation of two gBlocks. 2. Transformation by floral dipping. 3. Screening T1 seeds with red fluorescence. 4. Genotyping and sequencing T1 plants with red fluorescence to identify candidates and harvest seeds from each individual plant. 5. Isolating Cas9-free T2 seeds without red fluorescence. 6. Genotyping T2 plants to obtain stable and heritable null mutants.

B. Generate the middle border of two-gBlocks

1. Design two gRNAs to target the same gene of interest (GOI) with CRISPRdirect (Naito *et al.*, 2015) (Figure 2).

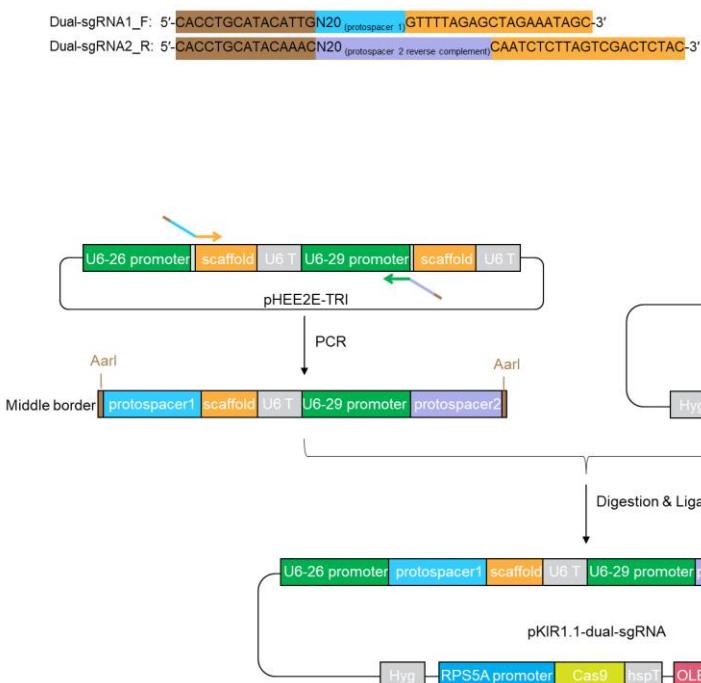


Figure 2. Overview of dual-sgRNAs cloning strategy. Schematic representation of the cloning strategy for the introduction of the middle border of two-gBlocks into the pKIR1.1 backbone. pHEE2E-TRI harbors two sets of gBlocks including gBlock1 (a U6-26 promoter, a 19 bp target sequence 1, a sgRNA scaffold, a terminator) and gBlock2 (a U6-29 promoter, a 19 bp target sequence 2, a sgRNA scaffold, a terminator). Therefore, pHEE2E-TRI can serve as a template to amplify the middle border (AarI-overhang1-protospacer1-scaffold-terminator-U6-29 promoter protospacer2-overhang2-AarI) using a pair of dual-sgRNA primers. After digestion of

pKIR1.1 plasmid and the middle border both by AarI, the middle border can be integrated into the linearized pKIR1.1 backbone to generate pKIR1.1-dual-sgRNA for plant transformation.

- Open the CRISPRdirect webpage (<http://crispr.dbcls.jp/>) and paste a target genomic sequence into text field in a FASTA format or a plain nucleotide sequence up to 10 kb.
Note: You can also enter an accession number (e.g., NM_001187) or genome location (e.g., hg19: chr7: 900000-901000) to retrieve sequence, or upload a sequence plain text file in a FASTA format or a plain nucleotide sequence up to 10 kb.
- NGG is selected on the ‘PAM sequence requirement’ panel.
- Select Thale cress (*Arabidopsis thaliana*) genome, TAIR10 as the organism.
- Click on ‘design’.
- Select two highlighted protospacer sequence by clicking on ‘show highly specific target only’ as shown in the screen shot below (Figure 3).

Results: [?](#)

Sequence name: NC_003071.7:8122119-8123076 Arabidopsis thaliana chromosome 2 sequence
 PAM sequence: NGG
 Specificity check: Thale cress (*Arabidopsis thaliana*) genome, TAIR10 (Nov, 2010)
 Time: 2020-08-01 00:12:09

• Highlighted target positions (e.g., **45 - 67**) indicate sequences that are **highly specific** and have fewer off-target hits.
 • Target sequences with '0' in '20mer+PAM' (in number of target sites column) are shown in gray.
 Such sequences may possibly span over exon-exon junctions, so avoid using these.
 • Target sequences with TTTTs are also shown in gray. Avoid TTTTs in gRNA vectors with pol III promoter.

show **highly specific** target only

Show 20 entries		Search:						
position	target sequence	sequence information				number of target sites ?		
start - end	20mer+PAM (total 23mer)	GC% of 20mer	Tm of 20mer	TTTT in 20mer	restriction sites	20mer +PAM	12mer +PAM	8mer +PAM
103 - 125	- [CGC]GTGAGTCGCCGCTCCCGTTTA [gRNA]	60.00 %	76.77 °C	-		1 [detail]	1 [detail]	44 [detail]
109 - 131	+ AGTCGGTCCCCTTAAATT[CGG] [gRNA]	50.00 %	73.81 °C	-		1 [detail]	1 [detail]	482 [detail]
110 - 132	+ GTGCCGTCCTCGTTAAATC[GGG] [gRNA]	55.00 %	73.37 °C	-		1 [detail]	1 [detail]	206 [detail]
114 - 136	- [CGG]TCCCGTTAAATCCTGGCTT [gRNA]	45.00 %	71.62 °C	-		1 [detail]	1 [detail]	80 [detail]
118 - 140	- [CCC]TTTAATTGGGGCTTTCGTCC [gRNA]	45.00 %	68.09 °C	-		1 [detail]	1 [detail]	412 [detail]
119 - 141	- [CCG]TTTAATTGGGGCTTTCGTCC [gRNA]	45.00 %	69.60 °C	-		1 [detail]	1 [detail]	281 [detail]
192 - 214	+ GTTGGCTAACCTTAGCGACGT[GG] [gRNA]	50.00 %	72.97 °C	-	BsiWI	1 [detail]	1 [detail]	40 [detail]
207 - 229	+ GACGTTGGCCAGCTTCGT[GCG] [gRNA]	60.00 %	77.72 °C	-	EaeI	1 [detail]	1 [detail]	42 [detail]
215 - 237	- [CCA]GCTTCGGTACGGCAGCGTTG [gRNA]	65.00 %	79.08 °C	-	MscI	1 [detail]	1 [detail]	51 [detail]
216 - 238	+ CAGCTTCCGTACGGCAGCGT[GCG] [gRNA]	65.00 %	80.43 °C	-	BsiWI	1 [detail]	1 [detail]	39 [detail]
217 - 239	+ AGCTTCCGTACGGCAGCGT[GCG] [gRNA]	60.00 %	79.61 °C	-	BsiWI	1 [detail]	1 [detail]	61 [detail]
222 - 244	- [CCG]TACGGCAGCGTGGGCTTAC [gRNA]	60.00 %	79.00 °C	-	BsiWI	1 [detail]	1 [detail]	13 [detail]
236 - 258	+ TGCGCTTACACTAACACGGT[GG] [gRNA]	50.00 %	74.83 °C	-		1 [detail]	1 [detail]	43 [detail]
316 - 338	+ CAGAACTGTCAGATCTGAAG[AGG] [gRNA]	45.00 %	66.11 °C	-	BglII	1 [detail]	1 [detail]	242 [detail]

Figure 3. gRNA selection. Example screenshot for gRNA selection using the CRISPRdirect webpage.

*Note: One limitation is to avoid presence of an AarI restriction site on your protospacer sequence. In addition, target sequence can be selected from both DNA strands and should be devoid of TTTTs. The distance between the two sgRNAs depends on your expected deletion regions. Small deletions (<100 bp) can be induced with relatively high frequencies and large one (up to 120 kb) with low frequencies (Ordon et al., 2016) in *Arabidopsis*. The deletion area at 5' end or*

3' end of the non-coding region doesn't show a frequency difference in this sequence from screen shot (NC_003071.7:8122119-8123076 *Arabidopsis thaliana* chromosome 2 sequence). The 20 nt-protospacer sequence does not have to start with a G, because our linearized plasmids retain a G overhang at the 3' end of the U6 promoter that serves as the first G preferred for initiating transcription at the U6 promoter.

2. Amplify the middle border from the templet pHEE2E-TRI (Figure 2)
 - a. Order forward and reverse primers from idtDNA (<https://www.idtdna.com/>) and dilute them to a final concentration of 10 μ M.

Dual-sgRNA1_F: 5'-CACCTGCATACATTG**N₂₀**GTTTAGAGCTAGAAATAGC-3'

Dual-sgRNA2_R: 5'-CACCTGCATAC**AAAC****N₂₀**CAATCTCTAGTCGACTCTAC-3'

AarL: CACCTGC

20 nt-protospacer 1 sequence: **N₂₀**

20 nt-protospacer 2 reverse complement sequence: **N₂₀**

Overhang1 (ATTG) and overhang2 (AAAC) in yellow

Template specific forward sequence in orange

Template specific reverse sequence in green

Note: Here we take the first and the last gRNA designs in the screen shot as an example:

protospacer 1: TAAACGGGACGGCGACTCAC

protospacer 2: CAGAACATCGTCAGATCTGAAG

protospacer 2 reverse complement sequence: CTTCAGATCTGACGATTCTG

- b. PCR amplification with the following setup:

5x HF buffer	5 μ l
dNTP (10 mM)	1 μ l
Dual-sgRNA1_F (10 μ M)	1.25 μ l
Dual-sgRNA2_R (10 μ M)	1.25 μ l
pHEE2E-TRI (100 ng/ μ l)	1 μ l
Phusion	0.25 μ l
Water	15.25 μ l
total	25 μl

PCR program:

Initial denaturation	98 °C	30 s	
Denaturation	98 °C	10 s	
Annealing	57 °C	30 s	
Elongation	72 °C	17 s	
Final elongation	72 °C	5 min	
Storage	4 °C	∞	

} 35 cycles

- c. Run 5 µl PCR product on an agarose gel to check a band with the expected size 620 bp and clean up the rest PCR product using Wizard® SV Gel and PCR Clean-Up System.

C. Ligate the middle border into pKIR1.1 (Figure 2)

1. Digest and phosphorylate PCR product (the middle border)

- a. Digest the purified PCR product with AarI for 6 hrs at 37 °C.

10x AarI buffer	5.0 µl
Middle border	X µl (=1.5 µg)
AarI	1.5 µl
50x oligo	1.0 µl
Water	42.4 – X µl
Total	50 µl

Note: 50x oligo contains the AarI recognition sequence for stimulating AarI activity (Grigaitė et al., 2002). Both 10x AarI buffer and 50x oligo are supplied with AarI enzyme from Thermo Fisher.

- b. Phosphorylate the digestion mixture for 30 min at 37 °C in the following step, then clean up the mixture using Wizard® SV Gel and PCR Clean-Up System.

Digestion mixture	25 µl
10x T4 ligase buffer	2.83 µl
T4 Polynucleotide Kinase	0.5 µl

Note: Skipping the phosphorylation step will lower the ligation efficiency.

2. Digest and dephosphorylate pKIR1.1

- a. Digest pKIR1.1 with AarI for 6 h at 37 °C.

10x AarI buffer	5.0 µl
pKIR1.1	X µl (= 1.5 µg)
AarI	1.5 µl
50x oligo	1.0 µl
Water	42.4 – X µl
Total	50 µl

- b. Dephosphorylate the pKIR1.1 digestion mixture by 1 µl of phosphatase (CIP) for 30 min at 37 °C and for 10 min at 80 °C.

Digestion mixture	50 µl
CutSmart® Buffer (10x)	5.67 µl
CIP	1 µl

- c. Perform agarose gel electrophoresis with 5 µl of digestion mixture to test the digest. The expected size of digested pKIR1.1 is around 18.5 kb. Clean up the rest digestion mixture using Wizard® SV Gel and PCR Clean-Up System.

Note: We recommend using pKIR1.1 plasmid as negative control. The digested linear plasmid fragment runs more slowly with a larger band size and can therefore be distinguished from negative control.

3. Ligate the digested middle border with the linearized pKIR1.1 at 16 °C for 30 min to generate the final vector pKIR1.1-dual-sgRNA:

AarI-digested pKIR1.1 vector	X µl (50 ng)
AarI-digested middle border	X µl (5 ng)
10x T4 ligase buffer	1.0 µl
Water	X µl
T4 Ligase	1.0 µl
Total	10.0 µl

Note: Here we used vector: insert molar ratio 1:3.

4. Transform *E. coli* competent cells using 10 µl of ligation product and spread the transformed cells on LB agar plates with 100 mg/L Spectinomycin, then incubate the selection plates overnight at 37 °C (transformation protocol is provided by Clontech: http://www.takara.co.kr/file/manual/pdf/tr_PT5055-2.pdf).

5. Pick 8 colonies to verify the correct insertion (Figure 4) by colony PCR using forward primer Mlo 1938 and your own protospacer reverse primer with the following setup:

10x HotMaster™ Taq Buffer with Mg ²⁺	2 µl
dNTP (10 mM)	0.4 µl
Mlo 1938 (10 µM)	1 µl
Dual-sgRNA2_R (10 µM)	1 µl
HotMaster™ Taq DNA Polymerase	0.1 µl
Water	15.5 µl

Pick half of a single colony with a sterile pipette tip and swirl in the PCR reaction.

Use the other half for inoculation.

Total	20 µl
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PCR program:

Initial denaturation	94 °C	2 min	
Denaturation	94 °C	20 s	
Annealing	57 °C	20 s	35 cycles
Elongation	65 °C	1 min	
Final elongation	65 °C	5 min	
Storage	4 °C		

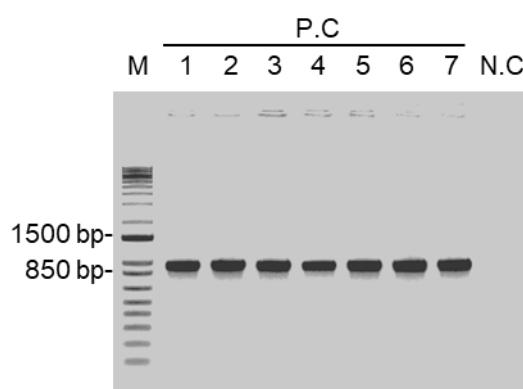


Figure 4. Electrophoresis of the colony-PCR products. The positive colonies show the correct insertion size with 868 bp. P.C: positive colony, N.C: negative colony.

6. Inoculate the positive colony in LB medium with 100 mg/L Spectinomycin and grow overnight.
 7. Extract the plasmid using the DNA plasmid kit.
 8. Sequence the plasmid and verify the sequence of the middle border insertion using primer Mlo 1938.
- D. *Arabidopsis* transformation with pKIR1.1-dual-sgRNA
1. Transform competent *A. tumefaciens* GV3101 cells with 1 µg of pKIR1.1-dual-sgRNA and spread the transformed cells on LB agar plates supplemented with 100 mg/L spectinomycin, 20 mg/L rifampicin, 25 mg/L gentamycin and 25 mg/L kanamycin (Höfgen and Willmitzer, 1988).
 2. After two days of growth at 30 °C, verify plasmid presence in at least three colonies by colony PCR as described before.
 3. Transform *Arabidopsis* plants using Agrobacterium-mediated T-DNA transfer with the floral dipping method (Clough and Bent, 1998).
- Note: Per construct, we usually transform at least ten *Arabidopsis* plants. We recommend removing of all present siliques before transformation to avoid excessive screening for transformation events afterwards.*
- E. Detect dual sgRNA-directed deletion by Cas9
1. Place the T1 seeds in a glass plate and pick up red fluorescent seeds with a slightly wet

toothpick under a Stereo Fluorescence Microscope using an EL6000 external light source with magnification 10 \times and DsRED filter set (excitation, 546/10 nm; emission, 600/40 nm) (Figure 5).

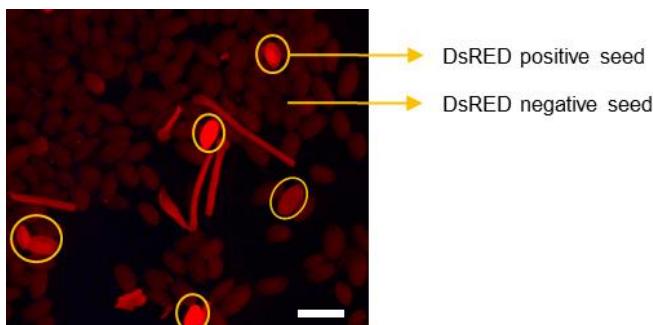


Figure 5. Visual screen for T1 seeds that harbor the CRISPR/Cas9 construct. pKIR1.1 vector can express the s.p. Cas9 protein driven by the RPS5A promoter, that allows high constitutive expression at all developmental stages including the germ cells. pKIR1.1 also contains an expression cassette of OLE1–TagRFP (red fluorescent protein) that exhibits red fluorescence in seeds. Therefore, the transformants containing CRISPR/Cas9 construct can be observed with red fluorescence in T1 seeds as indicated by circles. Scale bar = 1 mm.

Note: The transformation efficiency in this system is around 2.5%, which allows picking up 25 red T1 seeds within 10 min.

- Genotype the targeted deletion of T1 plants observed with red fluorescence in seeds by isolating leaf genomic DNA (gDNA) and subsequent PCR (Edwards *et al.*, 1991) using oligonucleotides flanking the deletion site (\pm 300-500 bp upstream and downstream) (Figure 6):

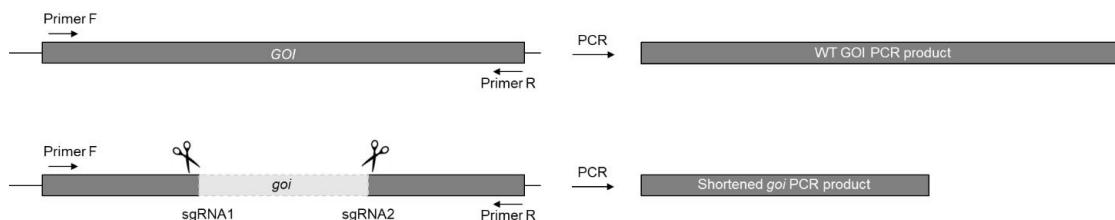


Figure 6. Genotyping the dual-sgRNA induced mutation on *GOI*. The large deletion of the *GOI* can be detected by PCR with a pair of primers flanking the deletion site (Primer F and Primer R). A shorter amplicon size can be detected in *goi* mutant compared to WT.

10x HotMaster™ Taq Buffer with Mg ²⁺	2 μ l
dNTP (10 mM)	0.4 μ l
Primer F (10 μ M)	1 μ l
Primer R (10 μ M)	1 μ l
Leaf gDNA	1 μ l (10 ng)
HotMaster™ Taq DNA Polymerase	0.1 μ l
Water	14.5 μ l

Total **20 µl**

PCR program:

Initial denaturation	94 °C	2 min]	35 cycles
Denaturation	94 °C	20 s		
Annealing	variable	20 s		
Elongation	65 °C	1 min/1 kb		
Final elongation	65 °C	5 min		
Storage	4 °C	∞		

F. Isolate Cas9-Free heritable mutation

1. Harvest T2 seeds from individual genotyped T1 plants for the second round of observation under the Stereo Fluorescence Microscope to select the desired progeny. T2 seeds that do not contain the CRISPR/Cas9 construct can be identified since they lack red fluorescence and isolated.
2. Genotype 10 Cas9-free T2 plants by PCR using oligonucleotides flanking the deletion site using phusion polymerase (Figure 7).

5x HF buffer	5 µl
dNTP (10 mM)	1 µl
Primer F (10 µM)	1.25 µl
Primer R (10 µM)	1.25 µl
Leaf gDNA	1 µl (10 ng)
Phusion	0.25 µl
Water	15.25 µl
Total	25 µl

PCR program:

Initial denaturation	98 °C	30 s]	35 cycles
Denaturation	98 °C	10 s		
Annealing	variable	30 s		
Elongation	72 °C	30 s/1 kb		
Final elongation	72 °C	5 min		
Storage	4 °C	∞		

3. Separate the PCR products on an agarose gel and extract the shortened *goi* band with the correct size from the gel using the Wizard® SV Gel and PCR Clean-Up System.
4. Sequence PCR product with Sanger sequencing and verify the sequence of the *goi* mutation. Usually heritable deletion events can be identified in plants lacking Cas9 activity after selection of seeds lacking the fluorescent reporter.

Data analysis

The sequencing data can be analyzed by alignment against the wild-type sequence using software SnapGene. Large deletion is generated in *goi* mutant of T2 (Figure 7).

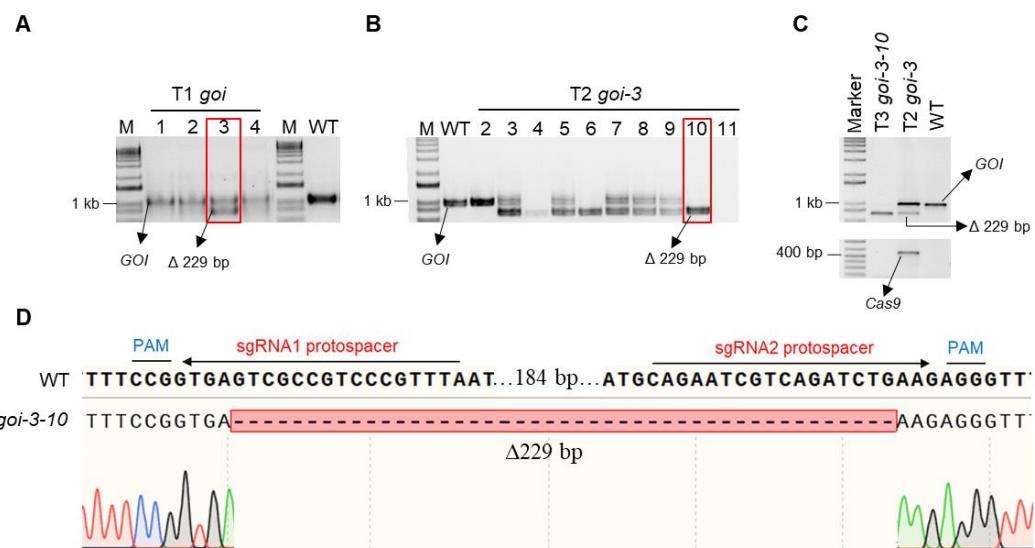


Figure 7. Representative chromatogram of PCR product from *goi* mutant. A. Genotyping of individual T1 plants that display red fluorescence. *goi-3* in red box shows two bands, WT GOI amplicon and *goi* amplicon. This representative *goi* mutant with an expected 229 bp deletion is generated by dual-sgRNA targeting at the genomic region Chr2: 8122228-8122453. PCR product with 1 kb in WT represents no deletion of the GOI. B. In the T2 generation, seeds from T1 *goi-3* are harvested and grown for screening Cas9-free plants. *goi-3-10* in red box (without red fluorescence in seeds) shows only the expected size of the *goi* amplicon. C. Bulked seedlings of *goi-3-10* in the T3 generation are genotyped. Top, only the expected *goi* amplicon is amplified, suggesting a heritable homozygous deletion. Bottom, no amplification of the Cas9-specific band (406 bp) validates fluorescence-based counter selection of the Cas9 transgene. *goi-3* in the T2 generation (no fluorescence selection) and WT are used as a positive control and negative control, respectively. D. Sequences of the representative deletion fragment of GOI ($\Delta 229$ bp). The alignment is generated with SnapGene. PAM are in blue, sgRNA protospacers are in red, and deleted bases are replaced by a dash in *goi-3-10*.

Note: It is mandatory to inspect the sequencing chromatograms carefully and check for the presence of overlapping peaks. Overlapping peaks are a sign of genetic heterogeneity in the sequenced sample.

Recipes

1. LB-liquid medium
10 g/L Bacto-tryptone

- 5 g/L yeast extract
5 g/L NaCl
2. LB-agar medium
10 g/L Bacto tryptone
5 g/L Bacto yeast extract
5 g/L Sodium chloride (NaCl)
15 g/L Bacto agar for plates
3. ½ MS-medium plates
4.4 g/L Murashige & Skoog medium
0.5 g/L MES
10 g/L Sucrose
Adjust pH to 5.7 with KOH
10 g/L plant agar for plates

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Competing interests

No competing interests.

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